Transient kinetics of ligand binding and role of the C-terminus in the dUTPase from equine infectious anemia virus

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Abstract Transient kinetics of the equine infectious anemia virus deoxyuridine 5′-triphosphate nucleotide hydrolase were characterized by monitoring the fluorescence of the protein. Rate constants for the association and dissociation of substrate and inhibitors were determined and found to be consistent with a one-step mechanism for substrate binding. A C-terminal part of the enzyme presumed to be flexible was removed by limited trypsinolysis. As a result, the activity of the dUTPase was completely quenched, but the rate constants and fluorescent signal of the truncated enzyme were affected only to a minor degree. We conclude that the flexible C-terminus is not a prerequisite for substrate binding, but indispensable for catalysis.

Key words: C-terminus; Deoxyuridine 5′-triphosphate nucleotide hydrolase; Deoxyuridine; Equine infectious anemia virus; Ligand binding; Pre-steady-state kinetics; Rate constant

1. Introduction

Deoxyuridine 5′-triphosphate nucleotide hydrolase (dUTPase, EC 3.6.1.23) is an enzyme in the pyrimidine-nucleotide metabolism. It catalyzes the hydrolysis of dUTP to dUMP and PP_i, a reaction crucial for the prevention of uracil incorporation into DNA. In addition to being essential for the viability of prokaryotic and eukaryotic species [1,2], its function is important for several viruses that have been found to encode dUTPases of their own [3–5]. Enzymes in the DNA metabolism have been used as targets for drugs against cancer and virus-inflicted diseases. Recent investigations have shown that dUTPase might be included among these targets [6–8]. Characterization of the enzyme, structurally and kinetically, would be a prerequisite for the design of such drugs.

With the exception of dUTPase from the vertebrate herpes viruses [9,10] and possibly from protozoa [11], dUTPase is a homotrimer with a molecular mass of around 50 kDa [12–14]. It has a low K_M (around 1 μM) and is highly specific for dUTP, discriminating against other pyrimidine nucleotides mainly by differences in binding strength [15–18]. The molecular background for the specificity has been investigated by X-ray crystallography [19,20]: the pyrimidine moiety of the substrate is bound in a hydrophobic cleft with considerably higher affinity for uracil than for other nucleotide bases. In the crystal structure of equine infectious anemia virus (EIAV) dUTPase in complex with a substrate analogue, a small conformational shift was seen in the cleft compared to the structure of the unliganded dUTPase [19]. In these structures, and in structures of dUTPase from other sources, a C-terminal portion that comprises about 10% of the polypeptide is disordered and has been considered to be flexible [13,19]. It contains several evolutionarily conserved residues, and has been ascribed a functional role [21,30].

Kinetic properties of dUTPase have previously been investigated by following the formation of product [15–18]. Here, we describe an approach not employed earlier to monitor the enzymic reaction, namely utilizing a shift in the fluorescence of the EIAV enzyme upon substrate binding. Using the stopped-flow technique, we have been able to study the transient kinetics of dUTPase ligand binding.

2. Materials and methods

2.1. Chemicals and enzymes

Chemicals were of analytical quality (Merck, Sigma, and Pharmacia). Nucleotides were purified before use [17,18]. Recombinant EIAV dUTPase was produced and purified as before [12] and stored at −20°C or in liquid N_2. C-terminal truncation (cf. [22]) of the EIAV dUTPase was carried out at 4°C, pH 6.9 (20 mM MES), 0.4 M NaCl and 0.1 M $MgCl_2$ using 42 μM dUTPase and 0.4 μM bovine pancreatic trypsin (Sigma), followed by analysis by SDS–PAGE (High Density PhastGel, Pharmacia). The truncated enzyme was purified on a mono-Q anion-exchange column (HR 5/5, Pharmacia) using a Bis-Tris-buffered gradient from pH 6.6, 30 mM NaCl, to pH 7.2, 0.5 M NaCl. Chromatographic peak fractions were analyzed by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI–MS) (Ludwig Institute, Uppsala, Sweden) using non-modified enzyme as standard. Protein concentration was determined spectroscopically at 280 nm using a molar extinction coefficient of 15 300 M^{-1} cm^{-1} [12].

2.2. Assay of dUTPase activity

The residual activity of C-terminally truncated EIAV dUTPase was assayed using [^{3}H]dUTP (Amersham) [23], as described by Bergman et al. [12]. The solution of truncated enzyme was used undiluted (12 μM), while the reference, unmodified enzyme was used very diluted (0.23 nM). Samples identical to those above, but with 0.01% sodium azide added, were run in parallel. The samples were incubated at room temperature for 24, 36 and 48 h. After subtraction of backgrounds, the ratio of dUMP over total nucleotide minus blank activity was plotted against incubation time. Samples containing sodium azide showed similar (with non-modified enzyme, even slightly lower) activity compared to those without, indicating that there were no artefacts in the results originating from contamination of biological activity, considering the long incubation time at room temperature.

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Abbreviations: dUPNPP, 2′-deoxyuridine 5′-(α,β-imido)triphosphate; dUTPase, deoxyuridine 5′-triphosphate nucleotide hydrolase; EIAV, equine infectious anemia virus; EL, enzyme–ligand complex

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2.3. Fluorescence measurements

Emission and excitation spectra were obtained with a Perkin-Elmer LS 50B spectrometer using 0.1–1 μM dUTPase. Time-resolved fluorescence measurements were carried out using an Applied Photophysics SX17.MV stopped-flow spectrophotometer with a dead time of 1.3 ms [24], 280 nm excitation wavelength, and a filter with a cut-off at 325 nm, allowing transmission of emitted light of longer wavelengths. With a maximum resolution of 80 data points per ms, 4000 data points were recorded for each reaction, using split timebase in the experiments where dUTP was expected to be hydrolyzed. One data set is the average of about 5–15 reaction traces.

The total enzyme concentration used was 1–2.5 μM. The concentration of active dUTPase during stopped-flow experiments was 0.3–1 μM, depending on the experiment, as calculated using k_{cat} for dUTP [18] and a V_{max} estimated from progression curves obtained at high C_s, both by dividing the integrated area over the curve with the maximum signal amplitude, and by estimating the time of the inflexion point of the substrate depletion phase. The two approaches gave equal results.

2.4. Evaluation of stopped-flow data

A mono-exponential equation was fitted to the progression curves, using the SX17.MV or SX18.MV software and data points from the dead time to seven half-times or longer. In the case of curves of dUTP hydrolysis, the starting value of the fitted function was fixed, assumed to equal the final fluorescence level. The observed rate constant thus obtained, k_{obs}, was plotted versus ligand concentration. The apparent rate constants, k_{ind} and k_{dep} (see Section 3.1), were obtained from the intercept and the slope of the plot, respectively, by linear regression (Microcal Origin v4.1) that takes the standard deviation in the k_{obs} values into account. The plots were linear (see Figs. 2 and 3), but to ensure that the relatively high enzyme concentration used did not affect the estimates of k_{dep} and k_{ind}, the data were also subjected to numerical integration using the software DYNAFIT [25] assuming the model $E+S \rightleftharpoons ES$, and using various values for enzyme concentration.

3. Results

3.1. Quenching of fluorescence upon ligand binding

On excitation with light of 280 nm wavelength, the EIAV dUTPase gives a fluorescence spectrum with a maximum around 340 nm (not shown), indicating a tryptophan residue as the source. Upon ligand binding (e.g. dUTP), emission above 325 nm decreases in intensity. This was exploited using a stopped-flow spectrophotometer for rapid mixing of enzyme and ligand solutions in order to monitor the formation of the enzyme–ligand complex (EL). With dUTP as the ligand, the first phase, the build-up of the EL concentration (Fig. 1a), is followed by the steady-state phase of substrate hydrolysis and ultimately, as the substrate is consumed, by a return to the intensity of the free enzyme (Fig. 1, inset).

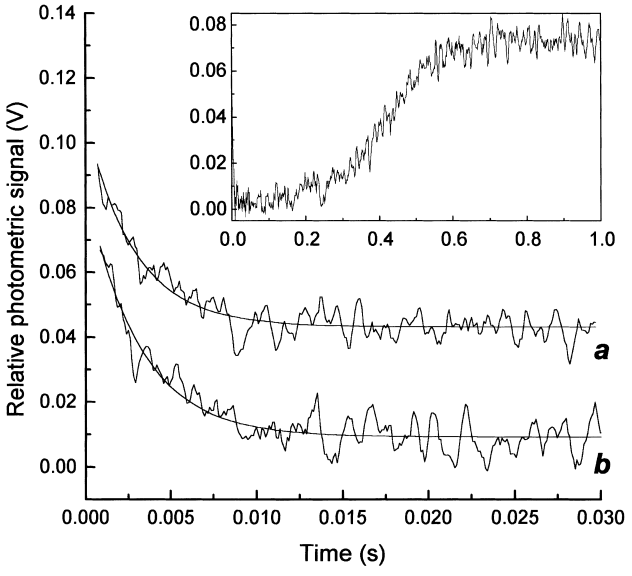

Fig. 1. Quenching of the fluorescence of dUTPase after mixing with dUTP in the stopped-flow cuvette. Inset: Fast formation of the EL, and return to the starting level after consumption of the substrate. a: Close-up of the ligand binding to functional dUTPase. b: Truncated dUTPase (Section 2.1). Fitting of a mono-exponential function to the data (e.g. curve a) results in an observed rate constant, k_{obs}, which is shown plotted against ligand concentration in Figs. 2 and 3.

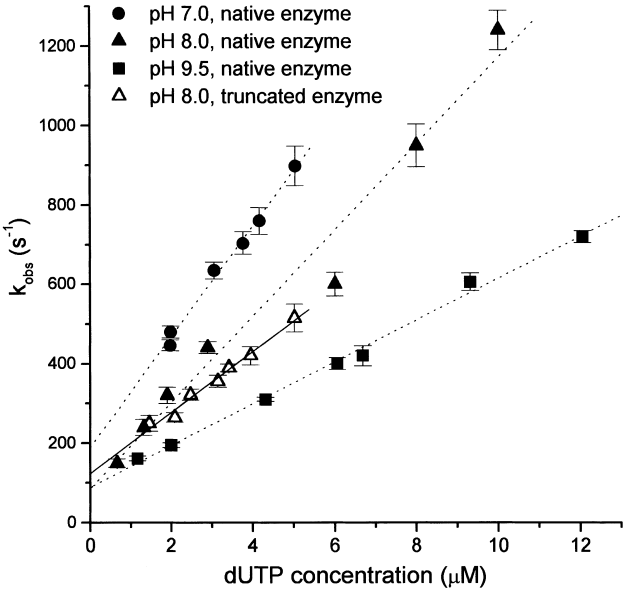

Fig. 2. Analysis of rate constants for the natural substrate dUTP: k_{obs}, obtained as in Fig. 1, as a function of dUTP concentration and pH (dashed lines). Also included: k_{obs} for dUTP at pH 8.0 with dUTPase truncated by the removal of 16 residues from the C-terminus (see Section 2.1) (solid line).

By fitting a mono-exponential equation to the pre-steady-state phase, a rate constant, k_{obs}, was obtained, which describes the rate of relaxation towards steady state or equilibrium. k_{obs} displays a linear dependence on ligand concentration for all ligand species investigated (Figs. 2 and 3). The relation can be described by Eq. 1, where the subscripts 'dep' and 'ind' denote dependence and independence, respectively, of ligand concentration.

$$k_{obs} = k_{dep} C_L + k_{ind} \quad (1)$$

If the event giving rise to the fluorescence signal was separate from the step of encounter of enzyme and ligand, k_{obs} should flatten out at the higher ligand concentrations used here. In attempts at a hyperbolic treatment of the experimental data, such a saturation could not be seen. Thus, any event between encounter and fluorescence shift is too fast to be relevant to the interpretations here. This conclusion is supported by the fact that k_{dep} for various ligands (see Sections 3.2 and 3.3) is in the same range as the association rate constants for other nucleotide binding enzymes (see Hiromi [26] for a compilation). Thus, a scheme representing the reaction can be written as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad (2)$$

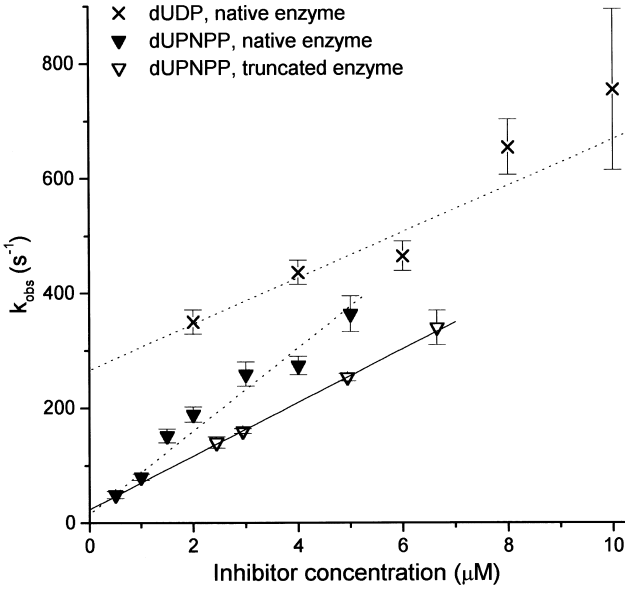

Fig. 3. Analysis of rate constants for non-hydrolyzable substrate analogues: k_{obs}, obtained at pH 8.0 using different concentrations of dUDP and dUPNPP [27] with native dUTPase (dashed lines), and of dUPNPP with truncated (Section 2.1) enzyme (solid line).

The slope of a plot of Eq. 1, k_{dep}, is taken to represent the second-order rate constant, k_1, while the intercept, k_{ind}, would be constituted by $k_{-1}+k_{cat}$, the rate constants independent of ligand concentration:

$$k_{obs} = k_1\ C_L + k_{-1} + k_{cat} \quad (3)$$

Fast rearrangement steps between the first and second steps in Eq. 2 cannot be excluded. In the measurements with the non-hydrolyzable nucleotides dUDP and 2′-deoxyuridine 5′-(α,β-imido)triphosphate (dUPNPP) [27], and in the case where the catalytic activity of the enzyme had been abolished by truncation (see Section 3.4), step 2 in Eq. 2 vanishes, k_{cat} in Eq. 3 becomes zero, and k_{ind} equals k_{-1}.

3.2. dUTPase and dUTP

For dUTP at pH 8.0, k_{dep} was determined to be 106 ± 9 $\mu M^{-1}\ s^{-1}$ and k_{ind} $95 \pm 26\ s^{-1}$. The ratio of k_{ind} to k_{dep} is in agreement with the K_M value (Table 1) determined earlier by monitoring the protons formed in the hydrolysis of dUTP [18]. The signal amplitude from steady state to completion of the reaction displays a hyperbolic dependence on dUTP concentration with a value of the half-saturation constant similar to the K_M value (not shown). To use the model above, k_1 takes the value of k_{dep}, while k_{cat}, determined earlier to be $25\ s^{-1}$ [18], can be used to calculate k_{-1}. Subtraction from k_{ind} gives a value of $70 \pm 27\ s^{-1}$ for k_{-1}.

The rate constants for dUTP are pH-dependent (Fig. 2, Table 1), showing a lower k_{dep} and k_{ind} at pH 9.5 and higher values at lower pH (pH 7.0 and 6.3): at pH 6.3, the rate was too fast to be accurately determined, and consequently a pK_a could not be estimated. The ratio of k_{ind} to k_{dep}, however, does not change significantly with pH, except at pH 9.5, where the value is somewhat higher. That the ratio has the same value at pH 7 and pH 8 is consistent with determinations of K_M at steady state [17,18]. These studies also corroborate the increase at higher pH, and have attributed it to titration of the uracil moiety of the substrate. The pH dependence of the rate constants around neutral pH is, however, an unexpected novelty, and points to features of the active site of dUTPase that are yet unknown.

3.3. Ligands other than the substrate

We then tested several inhibitory ligands of which dUMP and dTTP were found to reach equilibrium or steady state too fast to be measured. dUDP and dUPNPP showed rate constants in the same order of magnitude as the values found for dUTP (Table 1, Fig. 3). In both cases (Table 1), the ratio of k_{ind} to k_{dep} is similar to the K_i previously determined [18], supporting the validity of the approach presented here. dUDP gives a signal amplitude of the same magnitude as dUPNPP and dUTP.

3.4. Properties of truncated EIAV dUTPase

In the X-ray structures of homotrimeric dUTPases available in the data banks [13,19,28][1], the around 16 residues long C-terminus is undefined, which would suggest that it is flexible. However, this stretch is important for activity [21,22] although the role it plays in the reaction has not been clarified.

We removed the flexible C-terminal arm of the EIAV dUTPase by limited trypsinolysis, following in essence the procedure described for the *E. coli* dUTPase by Vertessy [22]. After purification, the truncated protein has a well-defined molecular mass of 13 230 kDa (SDS–PAGE and MALDI–MS, not shown) indicating Lys119 to be the last residue.

Upon removal of the C-terminal peptide, activity decreases by a factor of about 10^5 according to the assay based on hydrolysis of [^{3}H]dUTP (see Section 2.2). The residual activity might originate from incompletely digested enzyme co-purified with the truncated form. Alternatively, dUTPase, devoid of its C-terminal arm, may indeed possess an intrinsic but minuscule hydrolytic activity. In contrast, for truncated *E. coli* dUTPase, Vertessy [22] reported a significant residual activity. The difference between the two truncated proteins is notable, since the kinetic properties of the intact enzymes are comparable [17,18].

Fluorescence stopped-flow measurements confirm that the truncated EIAV dUTPase lacks activity. However, the protein binds the ligands dUTP and dUPNPP (solid lines in Figs. 2 and 3) with k_{ind} and k_{dep} values (Table 1) similar to the ones obtained with the intact enzyme. The signal amplitudes (Fig. 1b) were also the same as obtained with the native enzyme, implying that the flexible arm does not cause the fluorescence shift. This is further supported by the fact that dUDP, which does not interact with the C-terminal arm [19,30], gives as high a signal with the native enzyme as the other nucleotides.

4. Discussion

In the present study, we found that binding of deoxyuridine nucleotides to the EIAV dUTPase causes a shift in fluorescence, which in the case of dUTP, the substrate of the enzyme, becomes reversed upon hydrolysis of the nucleotide. The observed shift could reflect a change in the enzyme structure intimately connected with the catalysis. This change may be

[1] EIAV, *Escherichia coli*, and feline immunodeficiency virus. The coordinates for the human dUTPase by Mol et al. [29] have not yet been deposited. Consequently, their finding that the C-terminal is ordered in complex with deoxyuridine nucleotide cannot be dealt with here.

Table 1
Pre-steady-state rate constants of ligand binding to EIAV dUTPase

Complex		k_{dep} (μM^{-1} s^{-1})	k_{ind} (s^{-1})	Calculated k_{ind}/k_{dep} (μM)	Steady-state K_M (μM)[e]
Functional dUTPase with					
dUTP[a]	pH 7.0	142 ± 9.4	183 ± 25	1.29 ± 0.20	1.1 ± 0.1
	pH 8.0	106 ± 9	95 ± 26	0.90 ± 0.25	1.1 ± 0.1
	pH 9.5	51 ± 1.3	95 ± 5.6	1.86 ± 0.12	–
dUPNPP[b,c]	pH 8.0	72 ± 7	15 ± 11	0.21 ± 0.16	0.6 ± 0.03
dUDP[b]	pH 8.0	40 ± 9	265 ± 39	6.6 ± 1.8	3.6 ± 0.1
Truncated dUTPase[d] with					
dUTP[a]	pH 8.0	79 ± 6	114 ± 19	1.4 ± 0.27	–
dUPNPP[b,c]	pH 8.0	47 ± 1	23 ± 3	0.49 ± 0.06	–

The constants k_{dep} and k_{ind} obtained for the fluorescence shift are shown to equal k_1 and $k_{-1}+k_{cat}$, respectively, in Eq. 2 (k_{ind} equals k_{-1}, when no product formation occurs, see footnotes b and d).
[a]Substrate.
[b]Non-hydrolyzable substrate analogue.
[c]Persson et al. [27].
[d]EIAV dUTPase deprived of its C-terminal 16-residue peptide, see Section 2.1.
[e]K_M and K_i determined at steady state by monitoring proton release from the hydrolysis [18].

related to that observed by X-ray diffraction upon binding of dUDP [19]. Intact enzyme and enzyme with the flexible C-terminal arm removed behave similarly with respect to fluorescence implying that the structural change is confined to the globular part of the enzyme not involving the arm. The source of the fluorescence signal may be the tryptophan residues (Trp52), one from each of the subunits in the trimer, located at the three-fold axis of the enzyme. According to the crystal structures (Dauter, personal communication, 1999) [19], the orientation of the tryptophan side chains changes slightly upon nucleotide binding. X-ray diffraction to atomic resolution could be useful for a closer analysis of the events taking place upon nucleotide binding and in attempts at an unequivocal assignment of the fluorophore. However, identification of the fluorophore is not a prerequisite for further analysis and interpretation of the rate constants obtained here.

The results of the present work are consistent with an earlier report of kinetic parameters (k_{cat}, K_M, K_i) for the EIAV dUTPase that were acquired by monitoring protons released in the substrate hydrolysis [18]. In addition, our data contribute to a more detailed picture of the dUTPase reaction. The rate constants obtained are the first ones describing events in the dUTPase reaction ahead of its rate-limiting step. As seen in Section 3.1, the rates observed at different ligand concentrations can be interpreted straightforwardly by use of a one-step binding model. The scheme for substrate binding and hydrolysis (Eq. 2) and its cognate rate equation (Eq. 3) are supported by the data and state that the constants k_{dep} and k_{ind} determined here (Table 1) are k_1 and the sum of k_{-1} and k_{cat}, respectively. By subtracting k_{cat} for dUTP [18] from k_{ind}, a value for k_{-1} is obtained (Section 2). The difference between k_{-1} and k_{cat} at pH 8 is consistent with approximately every fourth substrate molecule bound to the enzyme becoming hydrolyzed, which corroborates the notion of dUTPase as an efficient enzyme (cf. Larsson et al. [17]). Furthermore, the assessment of the constants k_1 and k_{-1} provides, for the first time, access to a dissociation constant for Mg-dUTP from a dUTPase–substrate complex (K_s). A value for K_s allows an estimate of the difference in binding strength of dUTP and dUDP, a substrate analogue often used in X-ray studies of dUTPase. Our data indicate that K_s for dUTP is merely some five times lower than the K_i for dUDP [18]. Thus, introduction of the γ-phosphate does not provide a substantial increase in binding strength. This finding is notable since, in the X-ray structure [19], a cluster of lysine residues is present in the region of the active site, where the γ-phosphate is thought to bind, and, conceivably, capable of contributing to binding.

The fact that the rate constants for native and C-terminally truncated enzyme show no major differences strongly argues that the arm is not required in the substrate binding step. A competition between the arm and the ligand entering the active site (which would be expected, were there a high occupancy of the arm over the active site) would have made k_1 increase when the arm is removed. Instead, truncation causes a small decrease in k_1 and a slight increase in k_{-1} for dUTP and dUPNPP. These changes in the values might be attributed to a reduced electrostatic attraction between the negatively charged substrate and the enzyme due to removal of the positively charged C-terminus (cf. Adolph et al. [24]). The view that electrostatic interactions are involved gets support from a comparison between k_1 for dUDP and for the substrate. The ligand with less charge has the lower k_1. In conclusion, without a significant change in either the rate constants or the thermodynamic dissociation constant (k_{-1} over k_1) upon truncation, the C-terminal arm cannot play a role in the substrate binding step of dUTPase.

In the literature, the arm has often been ascribed an advanced role in the dUTPase reaction [29,30]. It has been suggested that the binding of substrate would induce a conformational change, resulting in the arm forming a lid on top of the substrate that after hydrolytic cleavage of the substrate opens up to release the products [30], facilitating the product release by pulling the newly formed dUMP out of the active site [29]. Our data may support a different view. Removal of the arm affects the rates and strength of ligand binding only to a minor degree. Since the arm is essential for catalysis, its main function could be to bring residues with catalytic roles to the bound substrate and to participate in hydrolysis (step 2 in Eq. 2). Clearly, there is a need for further investigation of the structural and dynamic properties of the arm.

The activity of the truncated enzyme is reduced to a level close to zero. A complex of truncated enzyme and the natural substrate might therefore be able to be analyzed by X-ray studies. Since removal of the arm does not largely affect the thermodynamic dissociation constant, it is likely that such an approach would reveal structural features of the catalytic complex.

The method of fluorescence-monitored transient kinetics

applied on dUTPase, as described here, could have wider applications. The method detects binding independently of catalytic activity. Just like the truncated inactive variant of EIAV dUTPase investigated in the present study, genetically engineered variants of the enzyme could be characterized using the method described here, and the influence of individual amino acid side chains on substrate binding be analyzed.

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